Petite colony formation by *Listeria monocytogenes* and *Listeria* species grown on esculin-containing agar

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Several strains of *Listeria* species formed petite-sized colonies from parent stock cultures when grown on agar media containing 0.2 –1% (w/v) esculin. This was observed in *Listeria monocytogenes* (7/22 strains), *L. innocua* (1/3), *L. grayi* (1/1), *L. seeligeri* (1/3), and *L. welshimeri* (1/1), but not in *L. ivanovii* (0/1) and *L. murrayi* (0/1). This phenomenon was only observed on agar media that contained esculin. All petite isolates had biotyping profiles identical to their larger, normal-sized counterpart isolates. Normal and petite-sized isolates from two *L. monocytogenes* strains, Scott A and V7, were pathogenic to immunosuppressed white mice. On media containing 0.5% (w/v) esculin + ferric iron, *Listeria* cultures produced colony diameters intermediate in size between those of normal and petite cultures. When pregrown in glucose broth, all petite isolates demonstrated visible β-glucosidase (esculinase) activity within 5 min, while the normal-sized isolates showed β-glucosidase activity only after at least 20–70 min. This evidence suggests that cells forming petite colonies are β-glucosidase constitutive variants within the parent population, while cells that form normal-sized colonies are inducible for β-glucosidase (esculinase) activity. A possible role for the esculin hydrolysis product, esculetin, in causing petite colony formation is discussed.

Key words: *Listeria* species, petite colonies, esculinase, pathogenicity.

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Quelques souches de *Listeria* forment des colonies de petite taille lors de sous-cultures des souches parentales sur des géloses contenant 0,2% à 1% (p/v) d'esculine. Cette particularité a été constatée chez *Listeria monocytogenes* (7/22 souches), *L. innocua* (1/3), *L. grayi* (1/1), *L. seeligeri* (1/3) et *L. welshimeri* (1/1), mais pas chez *L. inavovii* (0/1) et *L. murrayi* (0/1). Ce phénomène ne se produit que sur les géloses contenant de l'esculine. Chaque isolat de petites colonies avait un biotype identique à sa contrepartie formant des grosses colonies de taille normale. Des isolats à colonies normales et à colonies petites dérivées de deux souches de *L. monocytogenes*, Scott A et V7, se sont avérées pathogènes chez la souris blanche immunosupprimée. Sur des milieux contenant 0,5% (p/v) d'esculine + fer ferrique, les colonies de *Listeria* sont de taille intermédiaire entre les colonies petites et normales. Si les souches sont pré-cultivées dans un bouillon glucose, tous les isolats de petites colonies démontraient une activité β-glucosidase (esculinase) en moins de 5 min alors que pour les colonies normales cette activité β-gluconidase ne se manifestait qu'après 20–70 min. Cette observation suggère que les cellules formant des colonies petites sont des variants de la souche parentale possédant une β-glucosidase constitutive alors que les colonies de taille normale possèdent une β-glucosidase (esculinase) inductible. On discute du rôle de l'esculétine, un produit d'hydrolyse de l'esculine, dans la formation des petites colonies.

Mots clés : espèce *Listeria*, colonies petites, esculinase pathogénicité.

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Introduction

The formation of petite colonies by *Listeria monocytogenes* was first observed during a study on the fate of *L. monocytogenes* cells added to manufactured yogurt. Plating inoculated yogurt samples on the esculin-containing listeria selective isolation agar of Siragusa and Johnson (1988) occasionally gave some considerably smaller colonies in a background of larger sized colonies. Owing to the public health threat from *L. monocytogenes* and the potential for foodborne transmission (Schlech et al. 1983; Fleming et al. 1985; Linnan et al. 1988), we decided to investigate the occurence of petite colonies.

The objectives of the research reported here were to determine the roles of esculin and ferric iron on petite colony formation by *Listeria* spp., and to compare the esculinase activities and pathogenicity of petite and normal *L. monocytogenes* variants.

Part of this research was previously presented (G. R. Siragusa, L. A. Elphingstone, and M. G. Johnson. 1989. Abstr. Annu. Meet. Am. Soc. Microbiol. Paper P-40. p. 325.).

Material and methods

Cultures

All cultures were stored as 100% glycerol suspensions at −20°C, and grown in tryptic soy broth + 0.5% (w/v) yeast extract (TSBYE broth) at 37°C for use. The parent strains used in this study and their sources were previously described (Siragusa and Johnson 1988).

Media and components

All media and components were purchased from Difco Laboratories (Detroit, MI, U.S.A.). Esculin and other fermentation assay carbohydrates were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Colony diameter measurements

Organisms were grown in TSBYE broth for 12–18 h, diluted, and either spread plated or streaked on predried agar plates. In either case, the colonies were well-isolated (<75 cfu/spread plate) so crowding would not occur that would influence the resulting diameter of the colony. Plate's were incubated for 36–48 h at 37°C. Colony measurements were made using a set of dial calipers. The diameters of at least five colonies of any one type were measured per plate. Statistical

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analysis of the colony diameters of petitie and normal isolates was preformed using Scheffe's one-way ANOVA and Student's *t*-test with the ABSTAT (Anderson-Bell Corp., Parker, CO, U.S.A.) statistical software package.

Serial transfer effect

Petite isolates were tested for the ability to retain petite colony character upon successive serial transfer into TSBYE broth. After growth for 24 h at 37°C, an aliquot was diluted and surface spread plated on TSBYE + 0.5% (w/v) esculin agar (TESC), incubated for 36–48 h, then examined for petite colony formation. Approximately 10 μL of culture was transferred to a fresh TSBYE tube and the procedure repeated a total of five times.

Identification and biotyping protocols

Parent cultures as well as petite- and normal-sized isolates of *Listeria* strains Scott A and V7 were identified according to the United States Food and Drug Administration (US-FDA) recommended methods (Lovett 1986). Additional biotyping of all petite and normal isolates was done using API (Sherwood Medical, Dallas, TX, U.S.A.) Rapid Strep and Rapid CH test strips. API Rapid CH is a carbohydrate utilization profiling system that assays fermentation of 49 different carbohydrates. Carbohydrate utilization assays were also performed using 96-well microtiter plates and a 96-point inoculator described by Fung and Hartman (1972). A master inoculum plate was made by adding 100 μL of 36-h-old TSBYE culture of each test organism to duplicate wells. This plate was the inoculum source used to inoculate another 96-well microtiter plate with 200 μL well of purple broth base (Difco) containing 0.5% (w/v) of the respective test carbohydrate. Wells of carbohydrate media were prepared in duplicate, inoculated, and then incubated at 37°C.

β-D-*Glucosidase assay*

Assays of cultures for β-D-glucosidase (esculinase) were performed using a modification of the procedure of Edberg et al. (Edberg and Bell 1985; Edberg et al. 1985). Substrate reagent was prepared by dissolving 0.05 g of *p*-nitrophenyl-β-D-glucopyranoside in 10 mL of 0.05 M Sorensen's phosphate-buffered saline, pH 7.5. Cells were grown in 5-mL volumes of trypticase soy broth containing 0.5% (w/v) of glucose, salicin, or esculin for 36 h at 37°C. Stationary phase cells were pelleted, decanted, and resuspended to a turbidity corresponding to a McFarland No. 1 standard. In a sterile microtiter plate, 125 μL each of substrate and cell suspension were dispensed in duplicate wells and duplicate plates, and then incubated at 37°C. Suspensions were monitored for a positive reaction by the appearance of a yellow color from the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside and the subsequent release of *p*-nitrophenyl. Control wells contained either substrate or cells brought to volume with Sorensen's buffer.

Mouse pathogenicity testing and LD_{50} determination

The pathogenic potential of normal, petite, and parent stock cultures were assayed by the method of Stelma et al. (1987). Female Swiss Webster mice (22–25 g) were preinjected with 200 mg/kg body weight of carageenan 24 h prior to intraperitoneal challenge with $4–7 \times 10^4$ cells of the viable test organism. Five mice per test culture were challenged and held as a group in cages set inside isolation units. The mice were monitored for death over a 7-day period. Death of three or more mice per group of five constituted a positive mouse pathogenicity test result. Upon death, swab samples of the aseptically removed liver and spleen were cultured on lithium chloride – phenylethanol – moxalactam (LPM) (Lee and McClain 1986), and Oxford agars (Curtis et al. 1989) at 37°C for 36–48 h to demonstrate the presence of the injected challenge agents in postmortem tissues. Isolates were confirmed as *L. monocytogenes* serotype 1/2*a* or 4*b* by US-FDA recommended methods (Lovett 1984). LD_{50} assays were performed according to the method of Stelma et al. (1987). Five mice were injected from each of five 10-fold dilutions of an approximately 2×10^4 cfu/mL cell suspension. Dosages were determined from viable cell counts from culture spread platings of the undiluted suspension. The cell dosage per mouse required for 50% lethality was estimated from data obtained 5 days after challenge using the method of Reed and Muench (1938).

TABLE 1. Effect of media and media components on formation of petite colonies by parent cultures of *L. monocytogenes* Scott A and V7

Agar medium[a]	Formation of petite colonies from parent stock cultures: V7	Scott A
LSI	+	+
LSI without:		
esculin	–	–
acriflavin	+	+
bromcresol purple	+	+
esculin and acriflavin	–	–
bromcresol purple and acriflavin	+	+
TSBYE	–	–
TSBYE plus:		
arbutin, 0.5%	–	–
salicin, 0.5%	–	–
esculin, 0.5%	+	+

[a]TSBYE, tryptic soy broth + 0.5% yeast extract; LSI, listeria selective isolation agar. For formulae see Siragusa and Johnson (1988).

TABLE 2. Average colony diameters of *L. monocytogenes* Scott A and V7 parent cultures and V7 petite culture grown on TSBYE agar with varying esculin content for 36–48 h at 37°C

Esculin, % (w/v)	Mean colony diameter (mm): Scott A N	Scott A P	V7 N	V7 P	V7 petite
0.025	2.43	—[a]	2.42	—	2.88
0.05	2.29	—	3.22	—	2.30
0.1	1.91	—	2.42	—	2.11
0.2	1.86	—	2.40	0.84	0.92
0.4	2.08	0.78	2.22	0.81	0.70
0.5	1.98	0.72	2.12	0.83	0.72
1.0	1.81	0.71	1.60	0.50	0.67

NOTE: Each value represents the average of 10 colonies (5 on each of 2 plates). In all cases where petite colonies occurred their diameters were found, by a Student's *t*-test comparison or Scheffe's test, to be significantly smaller ($p = 0.05$) than their normal-sized counterparts. N, normal-sized colony; P, petite-sized colony.

[a]A dash indicates that only one colony size was seen; no smaller, petite-sized colonies were observed.

Carrageenan- and buffer-injected mice were included as controls during each pathogenicity test.

Results and discussion

Occasionally, upon plating inoculated yogurt samples on listeria selective isolation agar (LSI) (Siragusa and Johnson 1988), there would appear considerably smaller and paler colonies (Figs. 1*a*, 1*b*). Petite-sized colonies were easily distinguishable from their normal-sized counterparts on esculin-containing agar and were generally 2.8–2.9 times smaller than normal-sized isolates. Our first impression was that these were either non-*Listeria* organisms or that the parent stock culture was contaminated. The parent stock cultures of Scott A and V7, when streaked on TSBYE and on other agars (Lee's, BHI, MAC (Columbia CNA agar + acriflavin + ceftazidime), and AC (TSBYE + acriflavine + ceftazidime) agars) (Siragusa and Johnson 1988), did not show petite colonies (Table 1). To determine which, if any, LSI agar components triggered this phenomenon, the indi-

TABLE 3. Summary of *Listeria* species and strains showing formation of petite colonies from parent cultures when grown on TSBYE agar containing 0.5% (w/v) esculin (TESC agar)

Species and strain	Origin	Petite colonies formed[a]
L. monocytogenes		
ATCC 15313	Rabbit	−
ATCC 35152	nk[b]	−
V7	Raw milk	+
V37	Milk	−
V37CE	Milk	+
Scott A	Human	+
Brie-1	Cheese	−
LCDC 81-861	Cabbage	+
Murray B	Human	+
F1057	Milk	−
F1109	Milk	−
F2379	Cheese	−
CAP-unk.	Human	+
171	Human	−
1-Pre5	Environmental[c]	−
3-Pre7-5	Environmental	−
4-Pre5-2	Environmental	−
4-Pre6-4	Environmental	−
4-Pre11-4	Environmental	−
S433	Poultry[c]	−
S434	Poultry	−
S437	Poultry	+
L. innocua		
LA-1	nk	−
ATCC 33090	Cow brain	+
4-Pre12-4	Environmental[c]	−
L. seeligeri		
LA-15	Environmental	+
MC1412	nk	−
SE-31	nk	−
L. ivanovii		
KC1714	nk	−
L. murrayi		
ATCC 25401	Vegetation	−
L. welshimeri		
ATCC 35897	Vegetation	+
L. grayi		
ATCC 19120	Feces	+

[a]Colonies read after 36–48 h incubation at 37°C.
[b]nk, not known.
[c]Isolates identified by the methods recommended by the US-FDA (Lovett et al. 1986).

vidual additives in LSI were eliminated or added pairwise to modified LSI agar (Table 1). It was shown that within LSI agar, the esculin component would trigger petite colony formation (Table 1).

The formation of petite colonies from a parent stock culture could also be triggered by including a minimum of 0.2–0.3% (w/v) of esculin in TSBYE agar (Table 2), depending on the strain (V7 or Scott A). Other conjugated sugars, salicin and arbutin, when incorporated into TSBYE agar did not result in petite colony formation.

All petite and normal isolates that were derived from hemolytic parent stock cultures were also hemolytic on 5% sheep blood agar. Some, but not all, *Listeria* species and strains tested formed petite colonies on TESC agar (Table3).

TABLE 4. Biotype profile of *L. monocytogenes* Scott A normal and petite isolates[a]

Test	Normal	Petite
Gram reaction	+ (short rods)	+ (short rods)
Catalase	+	+
Sheep blood stab	Weak β hemolysis	Weak β hemolysis
Urea	−	−
Nitrate reduction	−	−
Motility (27°C)	+	+
MRVP[2]	+/+	+/+
Triple sugar iron agar	A/A, no gas	A/A, no gas
CAMP—*S. aureus*	+	+
CAMP—*R. equi*	−	−
Hippurate	+	+
Pyrolidine arylamidase	−	−
α-Galactosidase	−	−
β-Glucuronidase	−	−
β-Galactosidase	−	−
Alkaline phosphatase	−	−
Leucine arylamidase	+	+
Arginine dihydrolase	−	−

NOTE: Both phenotypes were positive for amygdalin, D-arabitol, *N*-acetyl-D-glucosamine, arbutin, cellobiose, esculin, fructose, β-gentobiose, D-glucose, lactose, maltose, mannose, melezitose, α-methyl-D-glucoside, α-methyl-D-mannoside, rhamnose, salicin, trehalose, xylitol utilization. Both phenotypes were negative for adonitol, D-arabinose, L-arabinose, L-arabitol, dulcitol, erythritol, D-fucose, L-fucose, galactose, gluconate, 2-keto-gluconate, 5-keto-gluconate, glycerol, glycogen, inulin, inositol, D-lyxose, mannitol, melibiose, raffinose, ribose, saccharose, sorbitol, L-sorbose, starch, D-tagatose, L-turanose, xylose, β-methyl-xyloside utilization.
[a]Based on results obtained from US-FDA *Listeria* indentification protocols, and API Rapid Strep and API Rapid CH test kits.
[b]MRVP, methyl-red, Voges-Proskauer medium.

Several of the petite as well as the normal-sized colonies of *Listeria* spp. that appeared on TSBYE agar containing 0.5% (w/v) esculin (TESC agar) were biotyped. Petite islolates were found not to be contaminants but had identification and biotyping profiles that were identical to their normal-sized isolate counterparts when tested by US-FDA recommended protocols, API Rapid Strep, and API Rapid CM (Table 4).

Microtiter plate carbohydrate utilization profiles conducted according to the method of Fung and Hartman (1972) were similar for petite and normal isolates of the five other strains of *L. monocytogenes* and of the other *Listeria* spp. (Table 5). The carbohydrates tested were glucose, salicin, esculin, α-methyl-D-mannoside, α-methyl-D-glucoside, lactose, cellobiose, arbutin, rhamnose, and starch.

Petite isolates of *L. monocytogenes* Scott A and V7 were grown in TSBYE broth, then spread plated on TESC agar daily for five successive transfers. Aliquots of each broth culture, when spread plated on TESC agar from 10^{-7}, 10^{-8}, and 10^{-9} dilutions showed no large or normal-sized colonies at any of the five serial transfers. The same cultures simultaneously spread plated on TSBYE agar showed the normal-sized colony phenotype, since no esculin was present to cause petite colony formation.

Petite colonies did not form when the TESC medium was supplemented with 0.5% (w/v) ferric citrate (TESC-Fe). Colony diameters formed on TESC-Fe agar were homogenous and intermediate in size to those formed on TSBYE agar and the petite colonies formed on LSI and TESC agars, with the exception of *L. monocytogenes* strain Murray B (Table 6).

Ferric iron complexes with esculetin (6,7-dihdroxycoumarin), the hydrolysis product of esculin, to form a black

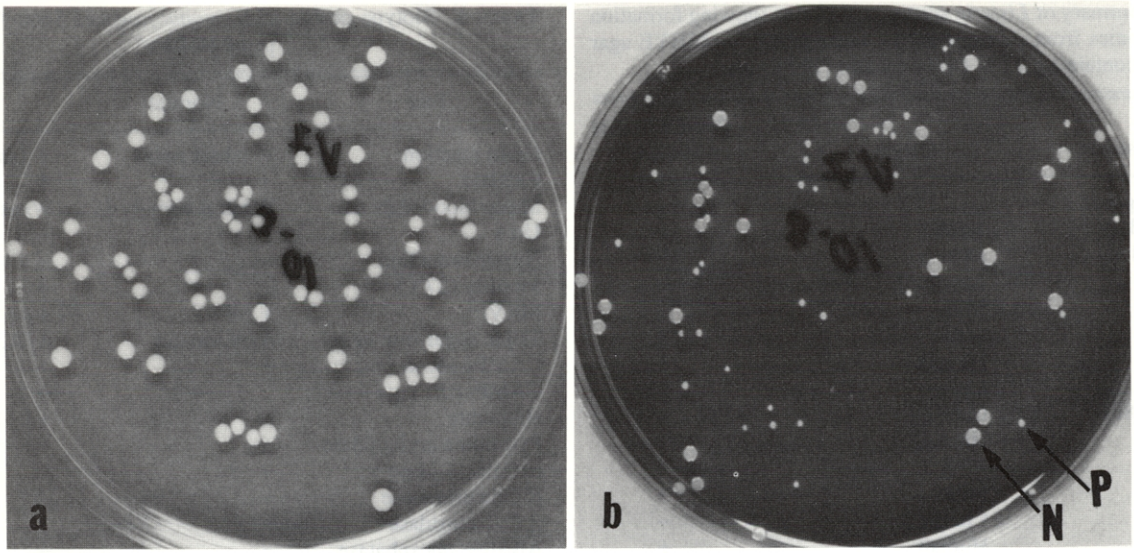

FIG. 1. (*a*) Spread plate culture of *L. monocytogenes* strain V7 on TSBYE agar. (*b*) Spread plate culture of *L. monocytogenes* strain V7 from the same parent stock as in Fig. 1*a* on LSI agar (esculin containing) showing the normal-sized (N) and petite-sized (P) colony phenoptypes. Plates were incubated at 37°C for 36–48 h.

TABLE 5. Microtiter plate carbohydrate utilization profiles of petite (P) and normal (N) cultures of *Listeria*[a]

	Colony type	Acid production from:									
		STA	ESC	α-MM	α-MG	LAC	SAL	RHA	CEL	GLC	ARB
L. monocytogenes											
Scott A	N	−	+	+	+	+	+	+	+	+	+
	P	−	+	+	+	+	+	+	+	+	+
V7	N	−	+	+	+	+	+	+	+	+	+
	P	−	+	+	+	+	+	+	+	+	+
S437	N	−	+	+	+	w	+	+	+	+	+
	P	−	+	+	+	w	+	+	+	+	+
LCDC 81–861	N	−	+	+	+	+	+	+	+	+	+
	P	−	+	+	+	+	+	+	+	+	+
CAP–unk.	N	−	+	+	+	+	+	+	+	+	+
	P	−	+	+	+	+	+	+	+	+	+
Murray B	N	−	+	+	+	w	+	+	+	+	+
	P	−	+	+	+	w	+	+	+	+	+
V37CE	N	−	+	+	+	w	+	+	+	+	+
	P	−	+	+	+	w	+	+	+	+	+
L. innocua											
ATCC 33090	N	−	+	+	+	w	+	+	+	+	+
	P	−	+	+	+	−	+	+	+	+	+
L. welshimeri											
ATCC 35897	N	−	+	+	−	w	+	−	+	+	+
	P	−	+	+	−	−	+	−	+	+	+
L. grayi											
ATCC 19120	N	−	+	+	−	+	+	−	+	+	+
	P	−	+	+	−	+	+	−	+	+	+
L. seeligeri											
LA-15	N	−	+	−	+	w	+	−	+	+	+
	P	−	+	−	+	w	+	−	+	+	+

NOTE: +, positive, −, negative; w, weak reaction. STA, starch; ESC, esculin; α-MM, α-methyl-D-mannoside; α-MG, α-methyl-D-glucoside; LAC, lactose; SAL, salicin; RHA, rhamnose; CEL, cellobiose; GLC, glucose; ARB, arbutin.

[a]According to the method of Fung and Hartman (1972).

TABLE 6. Average diameters of colonies formed by parent cultures of *Listeria* strains demonstrating petite colony formation on agar media with and without esculin and added ferric iron

	Colony type	Average colony diameter (mm) on:			
		TSBYE	TESC–Fe	TESC[a]	LSI[a]
L. monocytogenes					
V7	N	2.94	1.50	1.60	1.66
	P	—[b]	—	0.83	0.86
V37CE	N	2.27	1.17	1.14	1.12
	P	—	—	0.79	0.65
Scott A	N	2.83	1.39	1.89	1.74
	P	—	—	0.94	0.82
LCDC 81–861	N	2.52	1.26	1.74	1.45
	P	—	—	0.79	0.78
CAP-unk.	N	2.33	1.59	1.18	1.78
	P	—	—	0.63	0.73
Murray B	N	2.19	2.24	1.77	1.93
	P	—	—	0.72	0.81
L. grayi					
ATCC 19120	N	3.76	2.76	1.49	1.44
	P	—	—	0.67	0.67
L. seeligeri					
LA-15	N	2.48	1.74	1.24	0.98
	P	—	—	0.43	0.41
L. welshimeri					
ATCC 35897	N	2.73	1.95	1.61	1.25
	P	—	—	0.64	0.73

NOTE: TSBYE, tryptic soy broth agar + 0.5% (w/v) yeast extract; TESC–Fe, TSBYE agar + 0.5% (w/v) esculin + ferric citrate. LSI, listeria selective isolation agar, containing 0.5% (w/v) esculin (Siragusa and Johnson 1988). TESC, TSBYE agar + 0.5% (w/v) esculin.

[a]Values are averages of 10 colonies (5 from each of 2 plates) measured after 36–48 h incubation at 37°C. On TESC and LSI agars the diameters of petite colonies was significantly smaller than the normal-sized colonies ($p = 0.05$) in paired comparisons by the Student's *t*-test.

[b]A dash indicates that only one colony size was seen; no smaller, petite-sized colonies were observed.

precipitate. The incorporation of iron salts into bile–esculin agar has been used as an indicator of esculin hydrolysis to differentiate clinically important *Streptococci* (Kontnick et al. 1977). Several esculin-containing listeria enrichment media and agars call for the incorporation of iron salts for the purpose of indicating the possible growth of *Listeria* spp. (Fraser and Sperber 1988; Smith and Archer, 1988; Brackett and Beauchat 1989; Curtis et al. 1989).

Since esculetin is formed by the β-glucosidase catalyzed hydrolysis of esculin, the minumum incubation times required for detectable β-glucosidase activities of normal and petite isolates were determined. When pregrown in glucose, petite isolates showed β-glucosidase activity within 5 min of incubation vs. 20–70 min for normal-sized isolates (Table 7). Both normal and petite isolates pregrown in esculin- or salicin-containing broth (esculinase inducing conditions) showed β-glucosidase activity within 5 min of incubation. These results indicate that the petite colonies have constitutive β-glucosidase activity, whereas normal-sized isolates showed a delayed, inducible pattern for this activity.

Other workers (Edberg 1977; Edberg and Bell 1985; Edberg et al. 1985; Mishkin and Edberg 1978; Trepeta and Edberg 1987) studied esculin hydrolysis in the presence of bile in *Escherichia coli, Streptococcus* spp., *L. monocytogenes*, and *Fusobacterium* spp. Edberg et al. (1985) demonstrated constitutive esculinase activity in the presence of sodium deoxycholate in 11 out of 11 *L. monocytogenes* isolates within 15 min in a rapid bile–esculin assay.

Our results suggest that the petite colonies arise from individuals within the parent population that are constitutive for β-glucosidase activity, while the normal isolates are inducible within the time frame of the non-growth-supporting β-glucosidase assay used in this research. Phenotypic variation within a parent population of *L. monocytogenes* has been reported previously (Pine et al. 1987). These workers showed that *L. monocytogenes* ATCC 35152 contained both hemolytic and nonhemolytic variants.

It is possible that the formation of petite colonies is a result of esculetin released by esculin hydrolysis having an inhibitory effect on colony formation. Ferric iron forms a complex with esculetin resulting in the formation of a black compound readily seen in agar or in broth cultures. If esculetin is the inhibitory effector molecule, then it is possible that ferric iron complexing this compound could lessen the amount of free uncomplexed esculetin available to inhibit cell growth, so colonies formed were larger than those formed on TESC agar without iron (Table 6).

The more rapid formation of esculetin from esculin by petite isolates with constitutive β-glucosidase activity on TESC agar could exert an inhibitory effect on the cells, thereby restricting their growth to a smaller colony size. Normal inducible isolates, on the other hand, would first utilize available glucose before

TABLE 7. β-Glucosidase activities of normal (N) and petite (P) cultures of *Listeria* pregrown in trypticase soy broth containing 0.5% (w/v) glucose, esculin, or salicin

		Minimum time (min) for positive β-glucosidase test[a]		
		Glucose	Esculin	Salicin
L. monocytogenes				
Scott A	N	40	5	5
	P	5	5	5
V7	N	30	5	5
	P	5	5	5
V37CE	N	>70	5	5
	P	5	5	5
LCDC81–861	N	40	5	5
	P	5	5	5
Murray B	N	40	5	5
	P	5	5	5
CAP-unk.	N	40	5	5
	P	5	5	5
S-437	N	30	5	5
	P	5	5	5
L. grayi				
ATCC 19120	N	70	5	5
	P	5	5	5
L. innocua				
ATCC 33090	N	60	5	5
	P	5	5	5
L. seeligeri				
LA-15	N	20	5	5
	P	5	5	5
L. welshimeri				
ATCC 35897	N	40	5	5
	P	10	5	5
Control wells		—	—	—

[a]According to the method of Edberg et al. (1985*a*, 1985*b*), modified as described in Materials and methods. Assay substrate was *p*-nitrophenyl-β-D-glucopyranoside.

hydrolyzing esculin, thereby allowing *Listeria* cells to form larger sized colonies than their petite counterparts. Any potential growth advantage by petite variants on defined substrates or foods containing β-glucosides will be the focus of future work.

The pathogenic potential of *L. monocytogenes* Scott A and V7 petite vs. normal-sized isolates and parent stock cultures was assayed. Both petite and normal isolates, as well as the parent stock cultures of *L. monocytogenes* Scott A and V7, were pathogenic to mice, with all phenotypes (petite, normal, and parent culture) tested killing at least 4 or 5 out of 5 mice within 3 days. *Listeria innocua* ATCC 33090 (normal, petite, and parent cultures) were all nonpathogenic to mice, with no deaths occurring within 7 days from any of the phenotypes injected. *Listeria monocytogenes* Scott A normal and petite isolates had similar LD_{50} values when assayed using immunocompromised mice (14 vs. 4 cells per mouse for normal vs. petite isolates, respectively, as calculated using the method of Reed and Muench (1938) after 5 days postinjection). Livers removed aseptically from mice injected with petite cultures yielded only petite isolates that were confirmed as being the same species and serotype as was injected. No deaths occurred among carageenan- and buffer-injected control mice.

Petite isolates of *L. monocytogenes* Scott A and V7 were as pathogenic as normal isolates to immunosuppressed mice. Collectively, our results indicate that petite colonies of suspected *L. monocytogenes* from food or clinical samples arising on media containing esculin should receive the same careful follow-up biochemical testing as normal-sized colonies.

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BRACKETT, R. E., and Beauchat, L. R. 1989. Methods and media for the isolation and cultivation of *Listeria monocytogenes* from various foods. Int. J. Food Microbiol. **8**: 219–223.

CURTIS, G. D. W., MITCHELL, R. G., KING, A. F., and GRIFFIN, E. J. 1989. A selective differential medium for the isolation of *Listeria monocytogenes*. Lett. Appl. Microbiol. **8**: 95–98.

EDBERG, S. C., and BELL, S. R. 1985. Lack of constitutive β-glucosidase (esculinase) in the genus *Fusobacterium*. J. Clin. Microbiol. **22**: 435–437.

EDBERG, S. C., PITTMAN, S., and SINGER, J. M. 1977. The use of bile–esculin agar for the taxonomic classification of the family Enterobacteriaceae. Antonie van Leeuwenhoek, **43**: 31–35.

EDBERG, S. C., TREPETA, R. W., KONTNICK, C. M., and TORRES, A. R. 1985. Measurement of active constitutive β-D-glucosidase (esculinase) in the presence of sodium desoxycholate. J. Clin. Microbiol. **21**: 363–365.

FLEMING, D. W., COCHI, S. L., MACDONALD, K. L., BRONDUM, J., HAYES, P. S., PLIKAYTIS, B. D., HOLMES, M. B., AUDURIER, A., BROOME, C. V., and REINGOLD, A. L. 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. N. Engl. J. Med. **312**: 404–407.

FRASER, J. A., and SPERBER, W. H. 1988. Rapid detection of *Listeria* spp. in food and environmental samples by esculin hydrolysis. J. Food Prot. **51**: 762–765.

FUNG, D. Y. C., and HARTMAN, P. A. 1972. Rapid characterization of bacteria, with emphasis on *Staphylococcus aureus*. Can. J. Microbiol. **18**: 1623–1627.

KONTNICK, C., VON GRAEVENITZ, A., and PISCITELLI, V. 1977. Differential diagnosis between *Streptococcus agalactiae* and *Listeria monocytogenes* in the clinical laboratory. Ann. Clin. Lab. Sci. **7**: 269–276.

LEE, W. H., and MCCLAIN, D. 1986. Improved *Listeria monocytogenes* selective agar. Appl. Environ. Microbiol. **52**: 1215–1217.

LINNAN, M. J., MASCOLA, L., XIAO, D.L., GOULET, V., MAY, S., SALIMEN, C., HIRD, R. W., YONEKURA, M. L., HATES, P., WEAVER, R., AUDURIER, A., PLIKAYTIS, B.D., FANNIN, S. L., KLEKS, A., and BROOME, C. V. 1988. Epidemic listeriosis associated with Mexican-style cheese. N. Engl. J. Med. **319**: 823–828.

LOVETT, J. 1984. *Listeria* isolation. *In* Bacteriological analytical manual. Chap. 29. 6th ed. Food and Drug Administration, September, 1987, Revision 4. Association of Official Analytical Chemists, Arlington, VA. pp. 29.01–29.09.

MISHKIN, A., and EDBERG, S. C. 1978. Esculin hydrolysis reaction by *Escherichia coli*. J. Clin. Microbiol. **7**: 251–254.

PINE, L., WEAVER, R. E., CARLONE, G. M., PIENTA, P. A., ROCOURT, J., GOEBEL, W., KATHARIOU, S., BIBB, W. F., and MALCOLM, G. B. 1987. *Listeria monocytogenes* ATCC 35152 and NCTC 7973 contain a nonhemolytic, nonvirulent variant. J. Clin. Microbiol. **25**: 2247–2251.

REED, L. J., and MUENCH, H. A. 1938. A simple method for estimating fifty percent endpoints. Am. J. Hyg. **27**: 493–497.

SCHLECH, W. F., LAVIGNE, P. M., BORTOLUSSI, R. A., ALLEN,

A. C., HALDANE, E. V., WORT, A. J., HIGHTOWER, A. W., JOHNSON, S. E., KING, S. H., NICHOLS, E. S., and BROOME, C. V. 1983. Epidemic Listeriosis—evidence for transmission by food. N. Engl. J. Med. **308**: 203–206.

SIRAGUSA, G. R., and JOHNSON, M. G. 1988. Persistence of *Listeria monocytogenes* in yogurt as determined by direct plating and enrichment methods. Int. J. Food Microbiol. **7**: 147–160.

SMITH, J. L., and ARCHER, D. L. 1988. Heat-induced injury in *Listeria monocytogenes*. J. Indust. Microbiol. **3**: 105–110.

STELMA, G. N., JR., REYES, A. L., PEELER, J. T., FRANCIS, D. W., HUNT, J. M., SPAULDING, P. L., JOHNSON, C. H., and LOVETT, J. 1987. Pathogenicity test for *Listeria monocytogenes* using immunocompromised mice. J. Clin. Microbiol. **25**: 2085–2089.

TREPETA, R. W., and EDBERG, S. C. 1987. Esculinase (β-glucosidase) for the rapid estimation of activity in bacteria utilizing a hydrolyzable substrate, *p*-nitrophenyl-β-D-glucopyranoside. Antonie Leeuwenhoek, **53**: 273–277.